

# Introduction of silencing-inducing transgenes does not affect expression of known transcripts

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**Abstract** While the RNA interference (RNAi) mechanism has only been discovered a decade ago, RNAi is now often used to study gene function by sequence-specific knockdown of gene expression. However, it is still unknown whether introduction of silencing-inducing transgenes alters the transcriptome. To address this question, genome-wide transcriptional changes in silenced and non-silenced backgrounds were monitored through microarray analysis. No significant transcriptional changes were detected when compared to the non-silenced control. This result was confirmed by real-time polymerase chain reaction analysis of genes known to be involved in RNA silencing. In conclusion, introduction of silencing-inducing constructs does not affect expression of known transcripts in other genes than in those homologous to the targeted ones. Consequently, when gene function is studied by RNAi, the transcriptional changes detected will specifically be the result of knockout of the gene of interest, at least for the genes present on the array used in our study. © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** RNAi; Sense post-transcriptional gene silencing; Inverted repeat silencing; Hairpin silencing; Microarray; Transcriptome; *Arabidopsis thaliana*

## 1. Introduction

Determining the function of every gene encoded in the genome is an important task required to understand the biology of a particular organism. For many genes, their function is inferred from the phenotypes observed after their mutations. Countless screens for mutants in a particular pathway have already been carried out and large libraries of T-DNA or transposon integration lines have been generated (<http://www.arabidopsis.org/links/insertion.jsp>) that represent an important resource for gene disruption in the model system *Arabidopsis thaliana* [1]. However, mutation of some genes

causes lethality or does not result in a clear phenotypic defect because of redundancy with closely related genes.

Additional tools to deduce gene function have been provided by the determination of the genome sequence [2]. For instance, gene function can be predicted based on homology with genes or conserved domains of known function. Nevertheless, these postulations still need to be confirmed by a functional assay or by knocking-out of the gene.

Sequence-specific knockdown of genes can be obtained by RNA interference (RNAi) [3]. During RNAi, double-stranded RNA (dsRNA) is formed and, subsequently, cleaved into approximately 21 nucleotide-long small interfering (si)RNAs that guide a silencing effector complex to complementary RNA molecules, with degradation of the target as a result [4,5]. RNAi can be induced efficiently by introduction of a hairpin construct that can form the dsRNA intermediate directly with homology to the gene of interest [6]. Several systems to perform functional analyses using RNAi have been developed [3,6,7] because RNAi has some important advantages when compared to classical mutagenesis. For instance, RNAi can be induced at a particular developmental stage or in particular cells or tissues when the appropriate promoter is used to drive expression of the hairpin construct. Therefore, these inducible RNAi systems also allow the study of genes whose knockdown results in embryo lethality [8–10].

When exploiting RNAi, phenotypic outcome and transcriptional changes observed are assumed to result from downregulation of the targeted gene. However, it has not been investigated whether introduction of a silencing-inducing construct causes transcriptional changes. Therefore, genome-wide transcriptional changes in a silenced versus non-silenced transgenic background were monitored by means of CATMA array analysis [11]. Silent transgenes without homology to genes encoded in the *Arabidopsis* genome were selected to avoid cosuppression and both the silenced and non-silenced backgrounds were allelic to rule out secondary effects. No transcriptional changes were detected. This result was confirmed by monitoring mRNA accumulation levels of a subset of genes known to be involved in RNA silencing in transgenic lines in which silencing was induced by: (i) the presence of a sense transcript; (ii) two T-DNAs integrated as an inverted repeat, resulting in convergently transcribed transgenes; and (iii) a hairpin construct. We conclude that the RNAi machinery is constitutive and that, consequently, expression of a silencing-inducing construct does not affect the transcription of known genes in a non-specific manner.

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**Abbreviations:** AGO, argonaute; DCL, dicer-like; dsRNA, double-stranded RNA; Fab, fragment antigen binding; GST, gene-specific tag; GUS,  $\beta$ -glucuronidase; P<sub>35S</sub>, 35S promoter; PCR, polymerase chain reaction; RDR, RNA-dependent RNA polymerase; RNAi, RNA interference; siRNA, small interfering RNA

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Homozygous and hemizygous Kd27 plants were obtained as described previously [12] and by crossing the Kd27 line with a non-transformed *Arabidopsis thaliana* (L.) Heyhn. C24 plant [13], respectively. KH15 and KH15d6 have been described previously [14] as well as P<sub>35S</sub>:GUS and P<sub>35S</sub>:GUS+HP [15].

Seeds were surface sterilized, germinated on selective Murashige and Skoog medium (Sigma–Aldrich, St. Louis, MO, USA), supplemented with 1% sucrose. Plants were transferred to soil 21 days after germination and grown at 20 °C, 70% humidity, on a 16-h light/8-h dark cycle with 110 µmol m<sup>-2</sup> s<sup>-1</sup> of white fluorescent light.

### 2.2. Determination of Fab and GUS accumulation levels

Leaf samples were harvested 35 days after germination for analysis of transgene expression. For determination of fragment antigen binding (Fab) accumulation levels in homozygous and hemizygous Kd27 plants, protein extracts were prepared from fresh plant material [12]. Total soluble protein content was determined with the BioRad Protein Assay [16] with bovine serum albumin as a standard and Fab enzyme-linked immunosorbent assay was performed [17].

To determine β-glucuronidase (GUS) levels in KH15, KH15d6, P<sub>35S</sub>:GUS and P<sub>35S</sub>:GUS+HP plants, protein extracts were prepared as described [14], total soluble protein content was determined as above, and GUS activity as described [18]. GUS activity levels were expressed as units of GUS protein relative to the total amount of total soluble extracted protein.

### 2.3. Microarrays

The CATMA v2.2 array used in this study consisted of 23688 features, including 22494 unique gene-specific tags (GSTs) from *Arabidopsis* [19], 768 positive and negative control spots (GE Healthcare, Little Chalfont, UK) and 426 blank spots. Design and synthesis of primary and secondary GST amplicons have been described elsewhere [19,20]. The GSTs that primarily matched (3') exons or the 3' untranslated region sequences and occasionally (2.9%) contained intron sequences, were purified and arrayed as described elsewhere [11]. The CATMA GST array was printed at the VIB Microarray Facility ([www.microarray.be](http://www.microarray.be)) and consisted of two mega-columns and 12 mega-rows, resulting in 24 blocks. Each block represented a set of spots printed with a single and identical print tip. Prior to hybridization, the slides were washed in 2× saline–sodium phosphate–ethylenediaminetetraacetic acid buffer, 0.2% sodium dodecyl sulfate for 30 min at 25 °C.

### 2.4. Target labeling and hybridizations

Leaf samples of 35-day-old KH15 and KH15d6 shoots were harvested and immediately frozen in liquid nitrogen. Total RNA was prepared from ground frozen leaf material with the TriZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Five micrograms of total RNA was reverse transcribed to double-stranded cDNA and further amplified as described [21] as well as the subsequent Cy3 and Cy5 labeling, hybridization, post-hybridization washing, and scanning [11]. All protocols are available at the VIB Microarray Facility web site (<http://www.microarrays.be>) for Cy3 labeling, Cy5 labeling, hybridization, and scanning.

### 2.5. Experimental design

The two genotypes were twice hybridized to each other in both directions, involving two biological and two technical replicates, ensuring dyes were balanced within genotypes.

### 2.6. Statistical analysis of the diallel expression data

The expression data were analyzed in two steps: (i) a within-slide analysis to model the variation associated with spatial (such as grid layout on the slide) and structural components (such as print order, differential dye responses to binding, and scanning) and to remove this as noise; and (ii) a between-slide analysis to estimate the differences between genotypes and their consistency. Modeling the dye

bias was fitted within the spatial linear mixed model framework for within-slide analysis with a cubic smoothing spline curve (spline(intensity)) as implemented in the GenStat [22] menu for microarray data analysis. Once the corrected log ratios,  $M_{ij}$  (where  $i$  indexes the slides and  $j$  the probes), were obtained, the differences between targets were analyzed as implemented in the GenStat menu.

### 2.7. Real-time PCR analysis

RNA was extracted with TriZol (Invitrogen) with leaf material of the same 35-day-old plants used for determination of Fab or GUS accumulation levels. Total RNA was treated with DNase RQ1 (Promega, Madison, WI, USA) and subsequently purified with phenol–chloroform extraction. Poly(dT) cDNA was synthesized from 1 µg of DNaseI-treated total RNA with Superscript II reverse transcriptase (Invitrogen) and quantified on an iCycler real-time polymerase chain reaction (PCR) detection system (BioRad, Hercules, CA, USA) with the qPCR core kit for SYBR Green I (Eurogentec, Seraing, Belgium). PCRs were carried out in triplicate. Relative expression levels were first normalized to *ACTIN2* expression and then to the respective non-silenced controls. Specific primer pairs were designed with Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA, USA): At3g18780/*ACTIN2* 5'-GTTGACTACGAGCAGGAGATGG-3' and 5'-ACAAACGAGGGCTGGAACAAG-3'; At1g48410/*AGO1* 5'-TCTACAGGGATGGAGTCAGTGAGGG-3' and 5'-AGCCTCGTGTGATGACGCTTCTG-3'; At3g49500/*RDR6* 5'-AGAACTCATCCCTCCAAC AG-3' and 5'-CCAAGTCTCATTTCGCCAAG-3'; At1g01040/*DCL1* 5'-GACACCAGA GACACTTCCAATG-3' and 5'-CCAAGTCTCATTTCGCCAAG-3'; At3g03300/*DCL2* 5'-CCTGGACTATAACCGACATG-3' and 5'-GTGCTTATGGAGATGA TGAGAG-3'.

## 3. Results

### 3.1. The presence of silencing-inducing transgenes does not alter genome-wide transcript levels

Integration of two T-DNAs as an inverted repeat, resulting in convergently transcribed transgenes, triggers silencing to varying degrees [23]. In line KH15, the two inversely repeated *GUS* transgenes were separated by a 732-bp palindromic sequence, and silencing was efficiently induced. *Cre*-mediated deletion of one of the transgene copies in line KH15d6 alleviated *GUS* silencing [23]. The silenced line KH15 and the non-silenced control KH15d6 were chosen for genome-wide analysis of gene expression, because the transgenes were integrated at the same genomic locus into both lines. Therefore, effects due to transgene integration could be excluded and genes differentially expressed after induction of silencing could specifically be identified.

GUS protein activity was determined in leaf samples and 3.2% and 3.8% residual activity was detected in KH15 when compared to KH15d6 for the two biological replicates used. Part of the same samples was utilized for microarray analysis with CATMA chips. Analysis of normalized gene expression data was performed with GenStat (see Section 2.6). At the decision criterion based on an observed fold change in expression, i.e., >2 in absolute value, combined with a  $P$ -value <0.001, no differentially expressed genes could be identified (Fig. 1). Further examination of the relative expression of genes known to be involved in RNA silencing showed no significant change in KH15 when compared to KH15d6 plants (Table 1). Thus, RNA silencing induced by transgenes integrated as inverted repeat does not affect genome-wide expression of any gene present on the CATMA array nor genes known to be involved in RNA silencing.

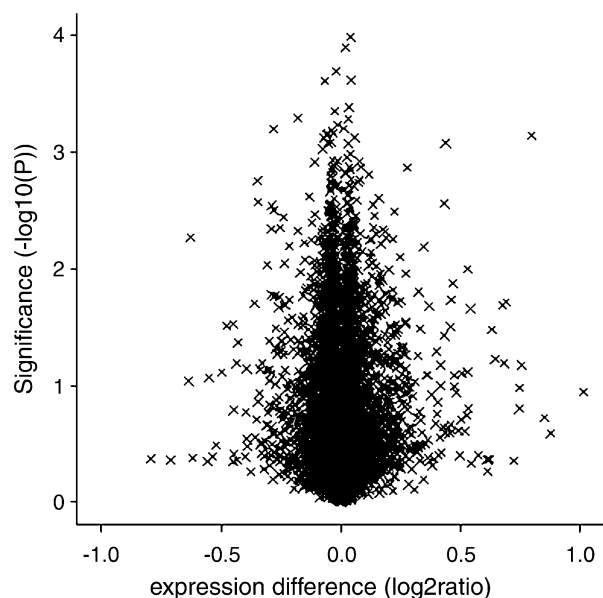


Fig. 1. Volcano plot contrasting the significance ( $-\log_{10}P$  on the ordinate) and the magnitude of the expression difference between KH15 and KH15d6 ( $\log_2$  on the abscissa). Each cross represents one of the 22494 genes.

### 3.2. Validation of microarray data by real-time PCR analysis

To confirm the results obtained with microarray analysis, real-time PCR was performed on a subset of genes known to be involved in RNA silencing, namely encoding the RNA-dependent RNA polymerase (RDR6), dicer-like1 (DCL1), DCL2, and Argonaute1 (AGO1) proteins. RDR6 encodes an RNA-dependent RNA polymerase, presumably required to synthesize dsRNA from 'aberrant' RNA molecules derived from silencing loci [24–26]. DsRNA is then cleaved into small interfering RNA (siRNAs) by a DCL protein [27]. Subsequently, the siRNAs associate with and guide AGO1 to complementary RNA molecules for degradation of the target [28]. For optimization of real-time PCR amplifications, 5-fold 1/5 dilution series of wild-type cDNA were amplified with the ACT2 (used as constitutive control), RDR6, DCL1, DCL2, and AGO1 primers to generate a standard curve. A linear re-

sponse ( $r^2 > 0.9$ ) was obtained, indicating that quantification was accurate at least over a dynamic range of 2.5 orders of magnitude (data not shown).

Before monitoring expression of RDR6, DCL1, DCL2, and AGO1, GUS accumulation in KH15 and KH15d6 was analyzed to confirm the silent state (Fig. 2). Subsequently, real-time PCR analysis of RDR6, DCL1, DCL2, and AGO1 mRNA accumulation levels was performed. No differences in expression levels of these genes were detected in KH15 when compared to KH15d6 (Fig. 3), confirming the results obtained with microarray analysis.

mRNA accumulation levels of these genes was also monitored in a line in which silencing was induced by the presence of a single copy of a sense transcript or a hairpin construct. Line Kd27 contains one K and one H T-DNA carrying the  $\kappa$ - and Fd-coding sequences in sense orientation, respectively. Accumulation of the  $\kappa$  and Fd translation products reconstitute Fab antibody fragments. When present in homozygous condition, the K T-DNA causes silencing of both the  $\kappa$ - and

Table 1

Relative expression levels of genes required for RNA silencing and paralogs in the silenced line KH15 versus the non-silenced control KH15d6 (ratio)

Gene	Code	Ratio	P-value
<i>AGO1</i>	At1g48410	0.97	0.118
<i>AGO2</i>	At1g31280	1.02	0.410
<i>AGO3</i>	At1g31290	1.00	0.874
<i>AGO4</i>	At2g27040	1.00	0.763
<i>AGO5</i>	At2g27880	1.01	0.640
<i>AGO6</i>	At2g32940	0.99	0.399
<i>AGO7</i>	At1g69440	0.98	0.444
<i>AGO9</i>	At5g21150	0.99	0.200
<i>AGO10/PNH</i>	At5g43810	1.02	0.349
<i>DCL1</i>	At1g01040	1.04	0.113
<i>DCL3</i>	At3g43920	1.01	0.378
<i>DCL4</i>	At5g20320	1.00	0.989
<i>HEN1</i>	At4g20910	1.00	0.569
<i>HYL1</i>	At1g09700	0.99	0.264
<i>RDR1</i>	At1g14790	1.00	0.917
<i>RDR2</i>	At4g11130	1.01	0.349
<i>RDR6/SGS2</i>	At3g49500	1.00	0.775
<i>SGS3</i>	At5g23570	1.01	0.735
<i>SDE4</i>	At1g63020	1.00	0.656

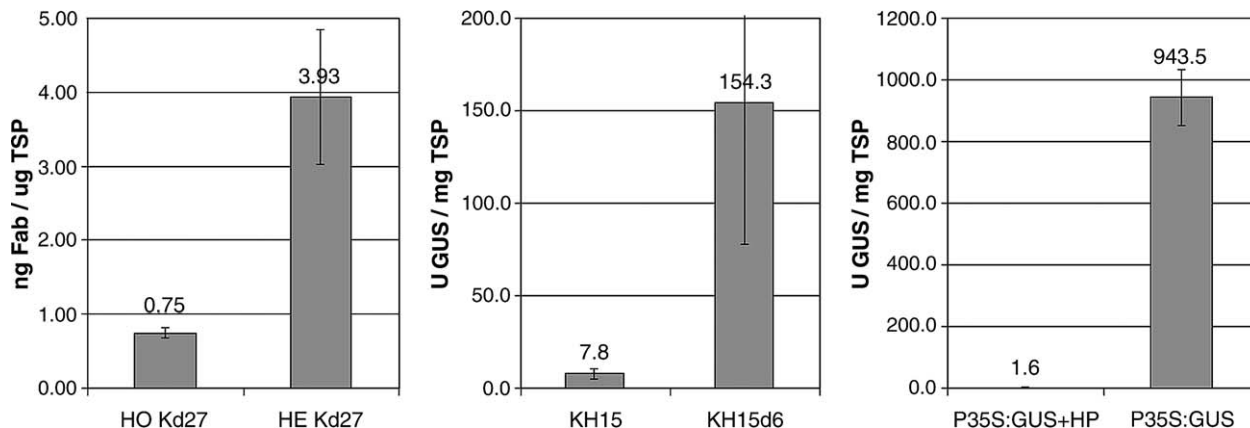


Fig. 2. Protein accumulation levels of transgenes in silenced and non-silenced backgrounds. In Kd27, Fab accumulation was determined by enzyme-linked immunosorbent assay. GUS activity in KH15 and P35S:GUS was determined spectrophotometrically with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide as a substrate. Error bars represent standard deviation of two biological repeats.

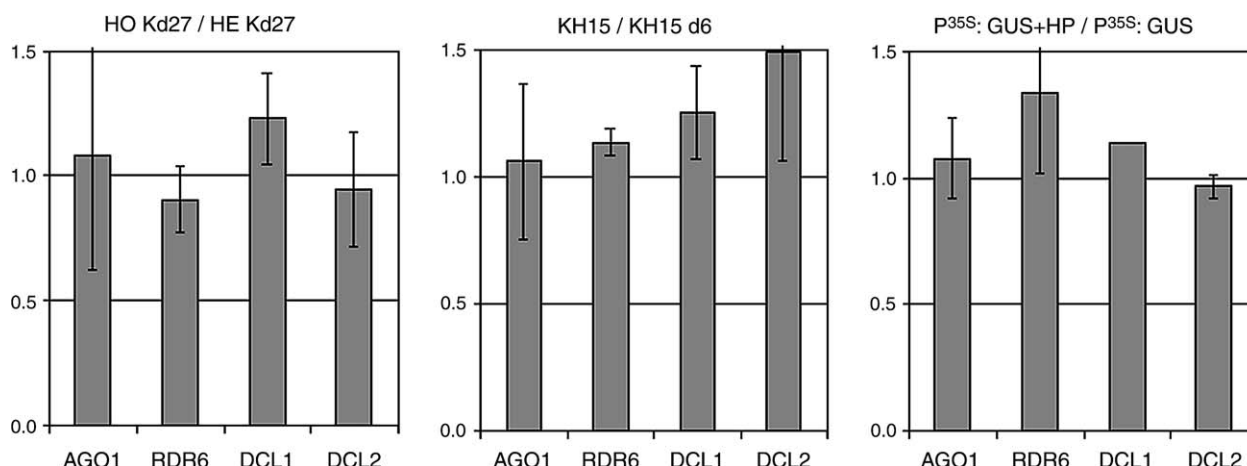


Fig. 3. Expression of *AGO1*, *RDR6*, *DCL1*, and *DCL2* in silenced versus non-silenced backgrounds. mRNA accumulation levels were determined by real-time PCR using *ACTIN2* as constitutive control. The fold difference of expression in the silenced plants when compared to the non-silenced control is shown. Error bars represent standard deviation of two biological repeats.

Fd-encoding sequences, which are homologous in the 3' untranslated region. In hemizygous condition Fab accumulation is high [13,17]. In the second transgenic line, high GUS activity obtained from integration of a single transgene carrying a  $P_{35S}:GUS$  expression cassette was strongly reduced after supertransformation with a hairpin-GUS construct under control of the  $P_{35S}$ -promoter. In the resulting  $P_{35S}:GUS+HP$  line, a single copy of both T-DNAs was integrated [15].

Monitoring transgene expression in the silenced transgenic lines showed that it was reduced when compared to the non-silenced controls (Fig. 2). Subsequently, mRNA accumulation of *AGO1*, *RDR6*, *DCL1*, and *DCL2* was assayed by real-time PCR. For HO and HE Kd27, and  $P_{35S}:GUS$  and  $P_{35S}:GUS+HP$ , mRNA accumulation levels of the silencing genes were very similar (Fig. 3). Student's *t*-test indicated that the differences in expression levels in the silenced lines and the non-silenced controls were not significant ( $P = 0.05$ ). In conclusion, no evidence for differential gene expression of *AGO1*, *RDR6*, *DCL1*, or *DCL2* could be found in plants with or without silencing of two different transgenes.

#### 4. Discussion

To investigate whether the integration of silencing-inducing transgenes influences transcript levels, we monitored genome-wide transcriptional changes in allelic silenced and non-silenced backgrounds. If induction of RNAi resulted in differential expression of a subset of genes, this would need to be taken into account when analyzing transcriptional changes after gene knockdown with RNAi to study gene function. No differential gene expression could be detected by microarray analysis in silenced transgenic plants in comparison to derived isogenic non-silenced control plants. This result was confirmed by monitoring transcriptional changes of a subset of genes known to be involved in RNA silencing by real-time PCR in these transgenic lines as well as in transgenic lines in which silencing was induced by gene dosage levels of a sense transcript or by a hairpin construct. The observation that introduction of a silencing-inducing construct does not affect transcript levels of known genes is important because it allows

us to conclude that changes in transcript levels observed after initiating RNAi to study gene function will be due to knock-out of the gene of interest and not to the introduction of an RNAi-inducing construct.

In this study, we made use of CATMA arrays [11] to monitor genome-wide transcriptional changes. Whereas these arrays have proven specificity and sensitivity [22], they cover mostly protein-encoding genes as well as some genomic regions with homology with open reading frames of transposable elements and pseudogenes. Therefore, we cannot rule out that we missed expression of protein-encoding genes or other sequences not present on the array used here. The use of TILLING arrays [28] that cover approximately 94% of the *Arabidopsis* genome sequence might be useful to further analyze the effect of introduction of silencing-inducing constructs on the transcriptome. However, for most routine analyses of differential gene expression, CATMA arrays, Agilent Arabidopsis2 oligonucleotide arrays, and Affymetrix ATH1 Gene-Chip probe arrays [29], which are similar to CATMA arrays in terms of coverage, sensitivity, and specificity [11], are used. Therefore, this study is an important control for the analysis of differential gene expression after RNAi-mediated knockout of gene expression.

These observations also have developmental consequences. An important role of RNA silencing in plants is to protect them against invading nucleic acids, such as viruses or transposons [30]. A paralog of *RDR6*, *AtRdP1*, which presumably functions as an RNA-dependent RNA polymerase to synthesize complementary RNA strands to generate dsRNA during transgene silencing [24,26], is involved in antiviral defense and could be required to generate dsRNA from viral sequences [31]. Because *AtRdP1* expression is induced after virus infection, *RDR6* might also be induced after introduction of foreign nucleic acids, such as silencing-inducing transgenes. However, expression of *RDR6* or other genes involved in RNAi was not altered after induction of transgene silencing.

Transposons that are mobile genetic elements might cause damage to the plant by excising and integrating into other regions of the genome [32] and their activity be suppressed by chromatin silencing [33]. Since methylation of the silenced *GUS* transgenes was observed [23], their suppression probably



also involves chromatin remodelling. In the derived non-silenced line, all methylation in the coding sequence was lost [23]. We also specifically focused on *RDR2*, *SDE4* (silencing-defective 4), *DCL3*, and *AGO4* expression because these genes are required for chromatin silencing [27,34,35]. However, these genes were not differentially expressed in KH15 and KH15d6. Thus, the RNA silencing machinery is constitutively expressed and might be required to continuously repress transposable elements present in the genome. Also, once invading nucleic acids are recognized, silencing will very quickly be established, thereby minimizing damage to the genome. Furthermore, the silencing machinery is involved in endogene regulation via microRNAs and siRNAs [4,36–38] and this gene control mechanism should not be disturbed upon genome stress or infecting nucleic acids.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.063.

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